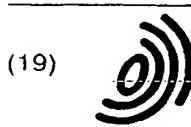


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(71) Applicant: Ajinomoto Co., Inc.  
Tokyo 104-0031 (JP)

(72) Inventors:  
• TAGUCHI, Seiichi  
Kawagoe-Shi, Saitama 350-1123 (JP)  
• MOMOSE, Haruo  
Kamakura-Shi, Kanagawa 247-0071 (JP)

(74) Representative: HOFFMANN - EITLE  
Patent- und Rechtsanwälte  
Arabellastrasse 4  
81925 München (DE)

(54) **PROCESS FOR PRODUCING MICROORGANISM-ORIGIN TRANSGLUTAMINASE**

(57) The present invention relates to a method of secretory production of transglutaminase by a microorganism.

The object of the present invention is to provide a method of produce a large amount of transglutaminase by causing Streptomyces bacteria to produce and secrete a large amount of transglutaminase.

The present invention is a method of producing a large amount of transglutaminase, comprising culturing

a Streptomyces bacterium harboring an expression plasmid containing a transglutaminase gene from actinomycetes and its native (naturally occurring) promoter, causing the bacterium to secrete protransglutaminase during the initial phase to the middle phase of culturing, and obtaining mature transglutaminase (active form) by cleaving and removing the pro9-structure, for example, with proteases derived from Streptomyces.

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**Description****Field of the invention**

- 5 [0001] The present invention relates to a method for secretory production of actinomycetes-derived transglutaminase by genetic recombinant techniques using the host-vector system of *Streptomyces lividans* (also referred to as *S. lividans* hereinafter. Transglutaminase). Transglutaminase produced and secreted according to the present invention is widely used in the food processing or medicines.
- 10 [0002] Transglutaminase which is produced and secreted according to the present invention is an enzyme which catalyzes the acyl transfer reaction of  $\gamma$ -carboxylamide groups located in the peptide chain of proteins. When the enzyme is reacted with a protein,  $\epsilon$ -( $\gamma$ -Glu)-Lys cross linking reaction, the substitution reaction from Gln to Glu by deamination of Gln can be occurred. Transglutaminase is used for producing gelatinized foods such as jelly, or yogurt, cheese, gelatinized cosmetics or improving the quality of meat (Publication of unexamined Japanese patent application (referred to as JP-Kokai hereinafter) No. 1-50382). It is also used for producing materials for thermostable microcapsules or carriers for immobilized enzymes. Thus transglutaminase is an enzyme highly useful in the industry.
- 15 [0003] It is previously known that there are animal-derived transglutaminase and bacteria-derived transglutaminase (microbial transglutaminase, which may be referred to as MTG herein after). The former is a calcium dependent enzyme and distributes in the animal organs, skin or blood and the like. The examples are, for example, human keratinocyte transglutaminase (M. A. Phillips et al. Proc. Natl. Acad. Sci. USA, 87, 9333 (1990)), human blood coagulation factor XIII (A. Ichinose et al., Biochemistry, 25, 6900 (1990)). Regarding to the latter, calcium independent one is discovered from *Streptoverticillium* bacteria. The examples are, for example, *Streptoverticillium griseocarneum* IFO 12776, *Streptoverticillium cinnamoneum* sub sp. *cinnamoneum* (which may be referred to as *S. cinnamoneum* herein after) IFO 12852 and *Streptoverticillium moharaense* (which may be referred to as *S. moharaense* hereinafter) IFO 13819 (JP-Kokai No. 64-27471). Peptide mapping and genetic structural analysis revealed that transglutaminases produced by these bacteria have no homology with the enzymes from animals (EP 0 481 504 A1).
- 20 [0004] Microbial transglutaminases (MTGs) had problems in the amount or yield because they are produced through purification procedures from the culture of bacteria such as above described bacteria. The production of transglutaminase using genetic engineering techniques is also attempted. Transglutaminase proteins and genes thereof are described in, for example, JP-Kokai No. 64-27471, Biosci. Biotech. Biochem., 58, 82-87 (1994), Biosci. Biotech. Biochem., 58, 88-92 (1994), JP-Kokai No. 5-199883, Biochimie, 80, 313-319 (1998), Eur. J. Biochem., 257, 570-576 (1998), WO 9606931 and WO 9622366, which describe the reports for the production by host-vector system such as *S. lividans*, *Aspergillus oryzae* or *Escherichia coli* (which may be referred to as *E. coli* hereinafter). A producing method using secretory expression in microorganisms such as *E. coli* or yeast (JP-Kokai No. 5-199883) and a method for producing functional MTG by expressing MTG as an inactive fused protein inclusion body, resolubilizing the protein inclusion body by protein denaturing agent and then reconstituting the protein (JP-Kokai No. 6-30771) are reported. However, in the secretory expression by conventional techniques using microorganisms, the problem of very poor expression level has been indicated. For secretory production of transglutaminase in *Streptomyces*, there is a report which describes an example including the specific description about secretory accumulation (Biosci. Biotech. Biochem., 58, 82-87 (1994); JP-Kokai No. 5-199883), where transglutaminase gene from *Streptoverticillium moharaense* was introduced into *Streptoverticillium lividans* as a host using genetic recombination techniques, however, the amount of the secretion was only about 0.1mg/l.

**Summary of the invention**

- 45 [0005] It is an object of the present invention to provide a method of producing a large amount of transglutaminase by secretory production of transglutaminase in *Streptomyces* bacteria.
- [0006] The method of the present invention is the method of producing a large amount of transglutaminase characterized in that a host-vector system for *Streptomyces* is used, where an expression plasmid which can highly express a gene in *Streptomyces* bacteria is constructed using transglutaminase gene from *Streptomyces*, that is, the signal peptide region and the pro-structural region and mature structural region and the native (natural) promoter region controlling the expression of transglutaminase, and the expression plasmid is introduced into a *Streptomyces* bacterium, the bacterium is cultured, and the bacterium is directed to secrete transglutaminase having additional pro-structural part (protransglutaminase) during the initial phase to the middle phase of culturing and then mature (active) transglutaminase is obtained in the late phase of culturing by cleaving and removing the pro-structure, for example, with proteases derived from *Streptomyces*.

**Brief description of Drawings****[0007]**

5      Figure 1. shows the construction procedure of the expression plasmid pUJ-MTG.

**Detailed Description of the Invention**

10     [0008] According to the present invention, a large amount of mature (active form) transglutaminase are obtained by using a Streptomyces bacterium as a host-vector system and generating an expression construct containing the gene for transglutaminase having the pro-structural part (pro-transglutaminase) linked to the down stream of the native promoter for transglutaminase gene, and by introducing the construct into a Streptomyces bacterium, expressing it and cleaving the extracellular secreted pro-transglutaminase, for example, with proteases produced by the same Streptomyces bacterium.

15     [0009] Secretory proteins are generally known to be translated as pre-peptides or prepro-peptides and then undergo post-translational modifications to generate mature proteins. Namely, it is generally known that secretory proteins are translated as pre-peptides or prepro-peptides, and then, the signal peptides (pre-part) thereof are cleaved to convert them into mature peptides or pro-peptides and the pro-parts of the pre-peptides are cleaved to generate the mature peptides. Transglutaminase is one of such proteins. As used herein, transglutaminase having both the signal peptide and the pro-part, namely the primary translational product, may be referred to as "prepro-transglutaminase" and transglutaminase having the pro-part but not the signal peptide may be referred to as "pro-transglutaminase". The pro-part of pro-transglutaminase may be referred to as "pro-structural part" or simply "pro-structure". As used herein, the "pro-structural part/pro-structure" of transglutaminase and the "pro-part" of the protein are used interchangeably. Thus, "pro-transglutaminase" may also be referred to as "transglutaminase with additional pro-structural part".

20     [0010] As used herein, a protein of which pro-part is "cleaved and removed" refer to a protein wherein one or more amino acid constituting the pro-part is removed by cleaving peptide bond, and it includes a protein having the identical N-terminal region to the native mature protein and also a protein having one or more additional amino acid derived from the pro-part at its N-terminal compared to the native protein or a protein having shorter amino acid sequence than that of the native mature protein. As used herein, "mature transglutaminase" and "active form transglutaminase" are used in the same meaning.

25     [0011] Generally, the genetic constructs used in the present invention are those having suitable sequences containing promoters, nucleotide fragments encoding prepro-transglutaminase and regulatory sequences required to express the intended proteins in Streptomyces bacteria, at the appropriate location. The Vector which can be used for the constructs are not limited and any vector can be used which can function in Streptomyces bacteria, and the vectors may be vectors which autonomously replicate extrachromosomally such as plasmids or may be vectors which can integrated into the bacterial chromosome. Plasmids from Streptomyces are preferable, and the examples include, for example, pIJ702 (J. Gen. Microbiol., 129, 2703-2714, (1983)) and the plasmids obtained by improving it.

30     [0012] The promoters which can be used in the present invention to express transglutaminase genes in Streptomyces bacteria are the native promoters of transglutaminase genes from Actinomycetes. However, in some cases the promoter region cannot be identified, because the consensus sequence is not established in Actinomycetes in contrast with E. coli. In such cases, the genetic fragment containing 5'-upstream region large enough to covering the structural gene for transglutaminase and the promoter region required to regulate its expression may be used.

35     [0013] Transglutaminase genes which can be used in the present invention are not particularly limited, and, for example, genes for secretory transglutaminase derived from *S. cinnamoneum* IFO 12852, *S. grosepcarneum* IFO 12776, *S. moharae* IFO 13819 and *Streptoverticillium lydicus* (WO96/06931) are preferable. Transglutaminase genes from *S. cinnamoneum* or *S. moharae* are particularly preferable, which are used with the native promoter of the respective transglutaminase genes.

40     [0014] The entire nucleotide sequence of transglutaminase gene from *S. cinnamoneum* IFO12852 containing the 5'-upstream region, which was identified by the inventors of the present invention, is shown in SEQ ID NO:1, and the amino acid sequence encoded by the nucleotide sequence is shown in SEQ ID NO:2. It was assumed that from amino acid at position 1 to position 32 in the amino acid sequence was the sequence for pre-part, from amino acid at position 33 to position 86 was the sequence for pro-part and from amino acid at position 87 to position 416 was the sequence for mature transglutaminase. The entire nucleotide sequence of transglutaminase gene containing the 5'-upstream region from *S. moharae* is also shown in SEQ ID NO:3, and the amino acid sequence encoded by the nucleotide sequence is shown in SEQ ID NO:4. From amino acid at position 1 to position 31 in the amino acid sequence is the sequence for pre-part, from amino acid at position 32 to position 76 is the sequence for pro-part and from amino acid at position 77 to position 407 is the sequence for mature transglutaminase.

45     [0015] Methods for introducing the genetic constructs used in the present invention are not particularly limited, and

the protoplast process (Gene, 39, 281-286 (1985); JP-Kokai No. 3-251182), electroporation process (Bio/Technology, 7, 1067-1070 (1989)) may be used. The thus obtained transformants may be cultured by using conventional methods and conditions. The medium for culturing these microorganisms may be a conventional medium, for example, a medium containing carbon sources, nitrogen sources and inorganic ions. It is preferable to add vitamins, organic micronutrients such as amino acids or natural materials such as polypeptone or yeast extract. As carbon sources, carbohydrates such as solubilized starch, glucose or sucrose, organic acids and alcohols are properly used. The culture is conducted under the aerobic condition for one day to 2 weeks appropriately maintaining pH ranging from 5.0 to 8.5 and the temperature ranging from 15°C to 37°C.

[0016] As nitrogen sources, ammonia gas, aqueous ammonia, ammonium salts and the like are used. As inorganic ions, magnesium ion, phosphate ion, potassium ion or iron ions and the like are properly used. By culturing the transformants under these conditions, prepro-transglutaminase is largely produced in the bacterial cells and extracellularly secreted as protransglutaminase and then pro-transglutaminase is cleaved in the medium by a protease which is produced and secreted by a Streptomyces bacterium itself under these condition, then mature (active form) transglutaminase is largely accumulated in the medium.

[0017] Transglutaminases produced and secreted according to the present invention can be purified from the medium after culturing, depending on their properties, using the methods well known to those skilled in the art. Transglutaminase may be purified, for example, by using known appropriate techniques such as ammonium sulfate precipitation or ethanol precipitation, as well as ion-exchange column chromatography, isoelectric focusing or gel filtration or the combination thereof, after removing cells, for example, by centrifugation.

### Examples

#### Example 1: Acquisition of transglutaminase gene from S. cinnamoneum IFO 12852

[0018] The sequence of transglutaminase from S. cinnamoneum CBS683.68 has been already determined (Biochimie., 80, 313-319 (1998)). The primer according to SEQ ID NO:5 and SEQ ID NO:6 are synthesized based on this sequence, and the region encoding mature transglutaminase was amplified by PCR from chromosomal DNA of S. cinnamoneum IFO 12852 prepared according to the method of Saito and Miura (Biochem., biophys., Act., 72, 619 (1963)). PCR was conducted using Pyrobest DNA polymerase (TAKARA Co.) under the condition according to its protocol.

**SEQ ID NO:5 5'-TCCGATGACCGGGAAACTCCTCCGCCGAG-3'**

**SEQ ID NO:6 5'-CGGCCAGCCTTGCTCCACCTTGGCGGGGGC-3'**

[40] [ Free text in the sequence listing]

[0019]

SEQ ID NO:5 :PCR primer for amplifying transglutaminase gene from S. cinnamoneum  
45 SEQ ID NO:6 :PCR primer for amplifying transglutaminase gene from S. cinnamoneum

[0020] The amplified 960bp DNA fragment are then reacted with [ $\alpha$ -<sup>32</sup>P]dCTP using Random Primer DNA Labeling Kit Ver.2 (Takara Co.), according to the protocol attached to the kit, to generate the DNA probe. Using the generated probe and the chromosomal DNA from S. cinnamoneum IFO 12852, Southern blot hybridization was conducted according to the conventional procedures such as those described in Molecular Cloning 2nd Edition (J. Sambrook, E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p9.31 (1989)), which confirmed that transglutaminase gene was present in the 3.5kb fragment which excised by BamHI restriction enzyme. The 3.5kb BamHI digested fragment from S. cinnamoneum IFO 12852 chromosomal DNA was recovered by agarose electrophoresis using EASYTRP Ver. 2 (Takara), inserted into BamHI site of pUC18, and introduced into competent Escherichia coli JM109 cells (Takara) 55 to generate a library. Using the previously prepared DNA probe, the library was screened by colony hybridization according to the conventional procedures such as those described in Molecular Cloning 2nd Edition (J. Sambrook, E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p9.31 (1989)) to obtain the strain harboring the plasmid where the fragment of transglutaminase gene was cloned. The plasmid was recovered from the strain, which was

designated as pB3.5.

[0021] The sequencing of the cloned fragment in pB3.5 confirmed that the transglutaminase gene from *S. cinnamoneum* IFO 12852 had almost identical nucleotide sequence to the transglutaminase gene from *S. cinnamoneum* CBS683.68. The transglutaminase gene was inserted such that the gene was transcribed in the direction from EcoRI to HindIII site within the multi-cloning site of pUC18. The sequencing was performed using Di-terminator Cycle Sequencing Kit (PE Applied Biosystems) and DNA Sequencer 373A (PE Applied Biosystems).

[0022] The determined nucleotide sequence and the amino acid sequence encoded by the nucleotide sequence are shown in SEQ ID NO:1 and SEQ ID NO:2, respectively. It was assumed that from amino acid at position 1 to position 32 in the amino acid sequence is the sequence for pre-part, from amino acid position 33 to position 86 is the sequence for pro-part and from amino acid at position 87 to position 416 is the sequence for mature transglutaminase.

#### Example 2: Construction of the expression plasmid for transglutaminase gene

##### 1) Acquisition of plasmid vector (pJ702)

[0023] pJ702 was prepared according to [J. Bacteriol., 162, 406-412 (1985); J. Bacteriol., 169, 1929-1937 (1987)]. More specifically, *Streptomyces lividans* 3131 (ATCC 35287)(J. Gen. Microbiol., 129, 2703-2714 (1983)) obtained by transforming *Streptomyces lividans* 66 with pJ702 was cultured under the following medium condition at 30°C for 2 days.

[YEME medium + 0.5% Glycine + 50µg/ml thiostrepton]	
0.3%	Yeast Extract
0.5%	Peptone
0.3%	Malto Extract
0.1%	Magnesium chloride
1.0%	Glucose
34.0%	Sucrose
0.5%	Glycine
0.1%	50mg/ml thiostrepton solution (Sigma : dimethylsulfoxide solution)(pH 7.0)

[0024] 200ml of the broth cultured under above condition was centrifuged (12,000g, 4°C, 10 min.), washed with 50mM Tris-HCl (pH(8.0) - 5mM EDTA-50mM NaCl, and the resulting bacterial cells were suspended in 10ml of Tris-HCl (pH8.0) - 10mM EDTA - 25% Sucrose ( TE-Sucrose). 2ml of TE-Sucrose containing 30mg/ml of Lysozyme (Sigma) and 4ml of 0.25M EDTA was added, the mixture was incubated at 37°C for 30 minutes, then 2ml of 20% SDS was added. 5ml of 5M NaCl was further added, gently mixed, and then, it was incubated overnight. After centrifugation (100,000g, 4°C, 40 min. ), 30% polyethylene glycol 6000 was added to the resulting supernatant at the final concentration of 10% and the mixture was incubated at 0°C for 4.5 hours. Then the mixture was centrifuged (900g, 4°C, 5 min.) and the resulting precipitation was dissolved in 10mM Tris-HCl (pH 8.0)-1mM EDTA - 50mM NaCl. To the mixture 1.2ml of the solution prepared to contain 16.8g of cesium chloride and 10mg/ml of ethidium bromide in 10mM Tris-HCl (pH8.0) - 1mM EDTA (referred to as "TE", hereinafter) was added, the residue was remove by centrifugation (1,300g, room temperature, 15 min.), and then the mixture was centrifuged (230,000g, 20°C, 12 hours). After centrifugation, the plasmid layer was drawn and isolated under UV lamp, the solution was repeatedly extracted with TE-saturated butanol 3 times to remove ethidium bromide. The solution was dialyzed against TE at 4°C overnight, extracted once with TE-saturated phenol and twice with chloroform/isoamyl alcohol, and then the aqueous layer was recovered. Then, 1/10 volume of 3M sodium acetate (pH5.2) solution and 2 volume of ethanol were added to the solution, and the mixture was allowed to stand at -80°C for 30 minutes. The precipitation was recovered by centrifugation (12,000g, 4°C, 15 minutes), washed with 70% ethanol, dried and dissolved in 200µl of TE. About 10µg of the plasmid was obtained.

##### 2) Construction of the expression plasmid

[0025] A shuttle vector was firstly constructed which can replicate both in actinomycetes (*Streptomyces*) host and *E.coli* host. Multicopy plasmid pJ702 for actinomycetes was digested with SacI and PstI to prepare large 5.1kb mel (tyrosine kinase gene) fragment from which the promoter region was deleted. pOSΔB-Ap1 (about 7.9kb)(Appl. Environ. Microbiol., 60, 3566-3572 (1994)) into which the fusion gene of protease inhibitor SSI (*Streptomyces subtilisin inhibitor*) gene and microbial peptide (apidaecin) gene was digested with HindIII and PstI to prepare the fragment of about 2kb.

Multicopy plasmid pUC18 (Takara) for Escherichia coli was digested with EcoRI, blunted with T4 DNA polymerase (Takara), and self ligated. Plamids which could not be digested with EcoRI were selected and the plasmids were digested with SacI and HindIII to prepare the 2.7kb fragment. Then, shuttle vector pUJS (about 9.8kb) was constructed by the ternary ligation of the 5.1kb SacI-PstI fragment from pIJ702, the 2kb HindIII-PstI fragment from pOSΔB-Ap1 and the 2.7kb SacI-HindIII fragment from pUC18.

[0026] pUJS was digested with HindIII and EcoRI to recover the large 8.6kb fragment. pB3.5 (about 6.2kb) containing transglutaminase gene from S. cinnamoneum IFO 12852 which was cloned according to (1) was digested with HindIII and EcoRI and the 3.5kb HindIII-EcoRI fragment was recovered. The 3.5kb HindIII-EcoRI fragment was inserted into HindIII and EcoRI site of pUJS to construct pUJ-MTG (about 12.1kb). The above described construction procedures are shown in Figure 1.

[0027] E. coli AJ13669 obtained by transforming E.coli with pUJ-MTG was deposited in the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology (1-3, Higashi 1 chome Tsukuba-shi Ibarakiken 305-8566, JAPAN) (The microorganism had been deposited as FERM P-17602 on October 14, 1999 and was transferred to the international deposit based on the Budapest Treaty as FERM BP-7287 on August 28, 2000).

#### Example 3: Transformation of S. lividans TK24

[0028] S. lividans TK24 is the strain derived from S. lividans 66, which is mounted a streptomycin resistance (GENETIC MANIPULATION OF STREPTOMYCES, A LABORATORY MANYAL: D.A. Hopwood et al., p266, 1985, The John Innes Foundation Norwich). This strain was provided from D.A. Hopwood (John Innes Institute, Colney Lane, Norwich NR4 7UH, U.K.) and is obtainable from D.A. Hopwood's laboratory. S. lividans Tk24 was treated to make protoplasts and transformed according to the method of [JP-Kokai No. 3-251182; Hunter, I.S., "DNA Cloning" A Practical Approach 2, Glover, D.M.(Ed.) IRL Press (1985), GENETIC MANIPULATION OF STREPTOMYCES, A LABORATORY MANUAL: D.A. Hopwood et al., p104, 1985, The John Innes Foundation Norwich]. More specifically, S. lividans was cultured in YEME medium + 0.5% Glycine at 30°C for two(2) days. 200ml of broth was centrifuged(1,300g, room temperature, 10 min.) and the resulting bacterial cells were suspended in 72ml of 0.35M sucrose.

[0029] The suspension was then centrifuged (1,300g, room temperature, 10 min.), incubated at 30°C for 2.5 hours, filtrated with absorbent cotton to remove residues. The resulting filtrate was centrifuged (1,300g, room temperature, 10 min.) and the precipitant was washed twice with 25ml of P-buffer and suspended in 1ml of P-buffer to prepare a protoplast suspension.

[P-buffer]	
TES[N-Tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid]	5.73g
Sucrose	103g
Magnesium chloride	2.03g
Potassium sulfate	0.5g
Calcium chloride	3.68g
Trace elements solution	2ml/L (pH7.4)

[0030] 1ml of 1% potassium phosphate solution per 100ml of P-buffer, which had been separately prepared, was added to P-buffer immediately before use.

#### [Trace elements solution]

[0031] It contains the followings per 1L of the solution:

Zinc chloride	40mg
Ferric chloride	200mg
Cupric chloride	10mg
Manganese chloride	10mg
Tetra sodium borate	10mg
ammonium molybdate	10mg

[0032] The transformation of S. lividans TK24 protoplast suspension with pUJ-MTG (about 12.1kb), transglutaminase gene expressing plasmid, was conducted as follows.

DNA solution (0.2μg/μl)	20μl
Protoplast suspension of <i>S. lividans</i> TK24	100μl
0.35M Sucrose	20μl
P-buffer containing 20% polyethylene glycol 1000	1.5ml

5 was gently mixed and allowed to stand for 2 minutes at the room temperature.

[0033] The mixture was centrifuged (1,700g, room temperature, 10 min.) and the pellet was collected, repeatedly 10 washed twice with P-buffer, suspended in 1ml of P-buffer and spreaded on the following R-2 agar plates.

[R-2 Agar plate]

[0034]

15 1) R-2/A

Potassium sulfate	0.5g/l
Magnesium chloride	20.2g/l
Calcium chloride	5.9g/l
Glucose	20.0g/l
Proline	6.0g/l
Casamino acid	0.2g/l
Trace elements solution	4ml
Agar	44.0g/l

.2) R-2/B

TES	11.5g/l
Yeast Extract	10.0g/l
Sucrose	203g/l (pH7.4)

35 3) 1% KH<sub>2</sub>PO<sub>4</sub>

1, 2) and 3) were separately prepared. R-2/A and R-2/B were mixed on the preparation of plates and 1ml of 1% KH<sub>2</sub>PO<sub>4</sub> solution per final volume of 200ml of the mixture was added. R-2 agar plates where the transformants were plated were incubated at 30°C for 18 hours. 1ml of P-buffer containing 200μg/ml of thiostrepton was poured on the pate to cover the entire surface of the agar and the plates were further incubated at 30°C for 7 days to obtain colonies. Plasmids were prepared from the obtained colonies to confirm that the intended plasmid was introduced.

#### Example 4: The expression of transglutaminase and secretory production

[0035] The transformant pUJ-MTG/*S. lividans* TK24 was cultured in 4ml of Triton-Soya Broth (DIFCO) liquid medium containing 10μg/ml of thiostrepton at 30°C for 3 days. 1ml of the culture was seeded into 100ml of the same liquid medium in 500ml Sakaguchi flask and cultured at 30°C for 2 weeks. The samples were taken sequentially from the culture and 10μl of the broth supernatant was subjected to SDS-PAGE and then to Western blot analysis using the anti-transglutaminase antibody described in JP-Kokai No.6-046855 according to the general method such as described in Molecular Cloning 2nd Edition (J. Sambrook; E.F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p18.60 (1989)). As a result, the secretory production (about 40-50mg/l) of transglutaminase with additional pro-structural part was found till about day 7 to 10 of culturing and then the yield of transglutaminase having almost the same molecular weight as that of mature transglutaminase, which can be the result of processing of pro-transglutaminase, increased on further culturing. At about week 2 in culturing, about 40-50mg/l of mature transglutaminase was accumulated.

[0036] Using the supernatant of broth at the phase where the amount of secretory production of transglutaminase with additional pro-structural part (protransglutaminase) was large, SDS-PAGE and semi-dry blotting to PVDF membrane were conducted ( Analysis of Protein Structure for Gene Cloning, Tokyo Kagaku Dojin (1993)). After blotting,

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the PVDF membrane was stained with Coomassie brilliant blue, de-stained and dried in air. The portion corresponding to pro-transglutaminase was excised and analyzed for the N-terminal amino acid sequence by the protein sequencer (Model 476A, Perkin-Elmer). The result confirmed the 10 amino acids sequence (Gly-Asp-Gly-Glu-Glu-Lys-Gly-Ser-Tyr-Ala-, SEQ ID NO:7) of pro-transglutaminase. This amino acid sequence differed from the sequence of pro-region indicated in Biochimie., 80, 313-319 (1998), but was identical to the amino acid sequence determined in Example 1 (SEQ ID NO:2).

[0037] According to the present invention, a large amount of transglutaminase can be obtained in the broth by directing Streptomyces bacteria to produce transglutaminase. Since transglutaminase accumulated in the broth according to the present invention is mature transglutaminase cleaved by proteases which are produced by the Streptomyces bacteria themselves, mature transglutaminase can be easily recovered from the broth on a large scale.

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## SEQUENCE LISTING

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&lt;110&gt; Ajinomoto Co., Inc.

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&lt;120&gt; A method of producing microbial transglutaminase

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&lt;130&gt; Y1H0984

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&lt;141&gt;

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&lt;150&gt; JP 11-295649

&lt;151&gt; 1999-10-18

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&lt;170&gt; PatentIn Ver. 2.1

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&lt;210&gt; 1

&lt;211&gt; 1461

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&lt;212&gt; DNA

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&lt;213&gt; Streptoverticillium cinnamoneum

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&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (151)..(1398)

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cggcggcagc cctccttgcc gccggcgca gacgcagga cggcgccggcc aaggccctga 60

5 gcggcagctc gtcgcaaacc cctccatgc gtcgtgtct cacatgcctt cgttcacga 120

10 ggcttcacca caagggagtt attgatttcc atg cac aaa cgt cgg aga ctt ctc 174

Met His Lys Arg Arg Arg Leu Leu

1 5

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55

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35 40 45

25

Ala Asp Asp Val Glu Ser Ile Asn Ala Leu Asn Glu Arg Ala Leu Thr

50 55 60

30

Leu Gly Gln Pro Gly Lys Pro Pro Lys Glu Leu Pro Pro Ser Ala Ser

35 65 70 75 80

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Ala Pro Ser Arg Ala Pro Ser Asp Asp Arg Glu Thr Pro Pro Ala Glu

40 85 90 95

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Pro Leu Asp Arg Met Pro Glu Ala Tyr Arg Ala Tyr Gly Arg Ala

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His Arg Asp Gly Lys Lys Gln Gln Met Thr Glu Glu Gln Arg Glu Lys

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5

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10

Pro Thr Asn Arg Leu Ala Phe Ala Ser Phe Asp Glu Asn Lys Tyr Lys

165 170 175

15

Asn Asp Leu Lys Asn Thr Ser Pro Arg Pro Asp Glu Thr Arg Ala Glu

20

180 185 190

25

Phe Glu Gly Arg Ile Ala Lys Gly Ser Phe Asp Glu Gly Lys Gly Phe

195 200 205

30

Lys Arg Ala Arg Asp Val Ala Ser Val Met Asn Lys Ala Leu Glu Asn

210 215 220

35

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40

Asn Asn Asn Asp Ala Leu Leu Arg Glu Asp Ser Arg Ser Asn Phe Tyr

245 250 255

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Ser Ala Leu Arg Asn Thr Pro Ser Phe Lys Glu Arg Asp Gly Gly Asn

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Tyr Asp Pro Ser Lys Met Lys Ala Val Ile Tyr Ser Lys His Phe Trp

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Ser Gly Gln Asp Gln Arg Gly Ser Ser Asp Lys Arg Lys Tyr Gly Asp

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Val Asn Phe Asp Tyr Gly Trp Phe Gly Ala Gln Thr Glu Ala Asp Ala

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Asp Lys Thr Thr Trp Thr His Gly Asp His Tyr His Ala Pro Asn Ser

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Asp Leu Gly Pro Met His Val His Glu Ser Lys Phe Arg Lys Trp Ser

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35

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Met Arg Ile Arg Arg Arg

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55				

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Arg Val Thr Pro Pro Ala Glu Pro Leu Asp Arg Met Pro Asp Pro Tyr

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45

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 30 Ile Tyr Ser Lys His Phe Trp Ser Gly Gln Asp Arg Ser Ser Ser Ala  
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His Tyr His Ala Pro Asn Gly Ser Leu Gly Ala Met His Val Tyr Glu

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10

Ser Lys Phe Arg Asn Trp Ser Glu Gly Tyr Ser Asp Phe Asp Arg Gly

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380

20

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385

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400

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Lys Val Lys Gln Gly Trp Pro

405

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&lt;212&gt; DNA

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&lt;213&gt; Artificial Sequence

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<223> Description of Artificial Sequence:PCR primer for  
amplification of transglutaminase gene from  
*S.cinnamoneum*

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30          <223> Description of Artificial Sequence:PCR primer for  
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80          5

85          10

#### Claims

- 90          1. A bacterium belonging to *Streptomyces*, wherein a gene construct containing a transglutaminase gene from actinomycetes and a promoter sequence controlling the transglutaminase gene is introduced into the bacterium.
- 95          2. The bacterium belonging to *Streptomyces* according to claim 1, wherein the actinomycetes is *Streptoverticillium cinnamoneum*.
- 100         3. The bacterium belonging to *Streptomyces* according to claim 1 or 2, wherein the bacterium belonging to *Streptomyces* is *Streptomyces lividans*.

4. A method of producing a pro-transglutaminase derived from actinomycetes, comprising the steps of culturing the bacterium belonging to Streptomyces according to any one of claims 1 to 3, and allowing the bacterium to secrete the pro-transglutaminase into a culture medium.
5. A method of producing a mature transglutaminase derived from actinomycetes, comprising the steps of culturing the bacterium belonging to Streptomyces according to any one of claims 1 to 3, allowing the bacterium to secrete a protransglutaminase derived from actinomycetes into a culture medium, cleaving the pro-structural part of the pro-transglutaminase by a protease derived from said bacterium belonging to Streptomyces and recovering the mature transglutaminase.
- 10 6. The method according to claim 4, wherein the pro-transglutaminase has the amino acid sequence from Glycine residue at position 33 to Proline residue at position 416 in the sequence of SEQ ID NO:2.
- 15 7. The method according to claim 5, wherein the mature transglutaminase has the amino acid sequence from Serine residue at position 87 to Proline residue at position 416 in the sequence of SEQ ID NO:2.

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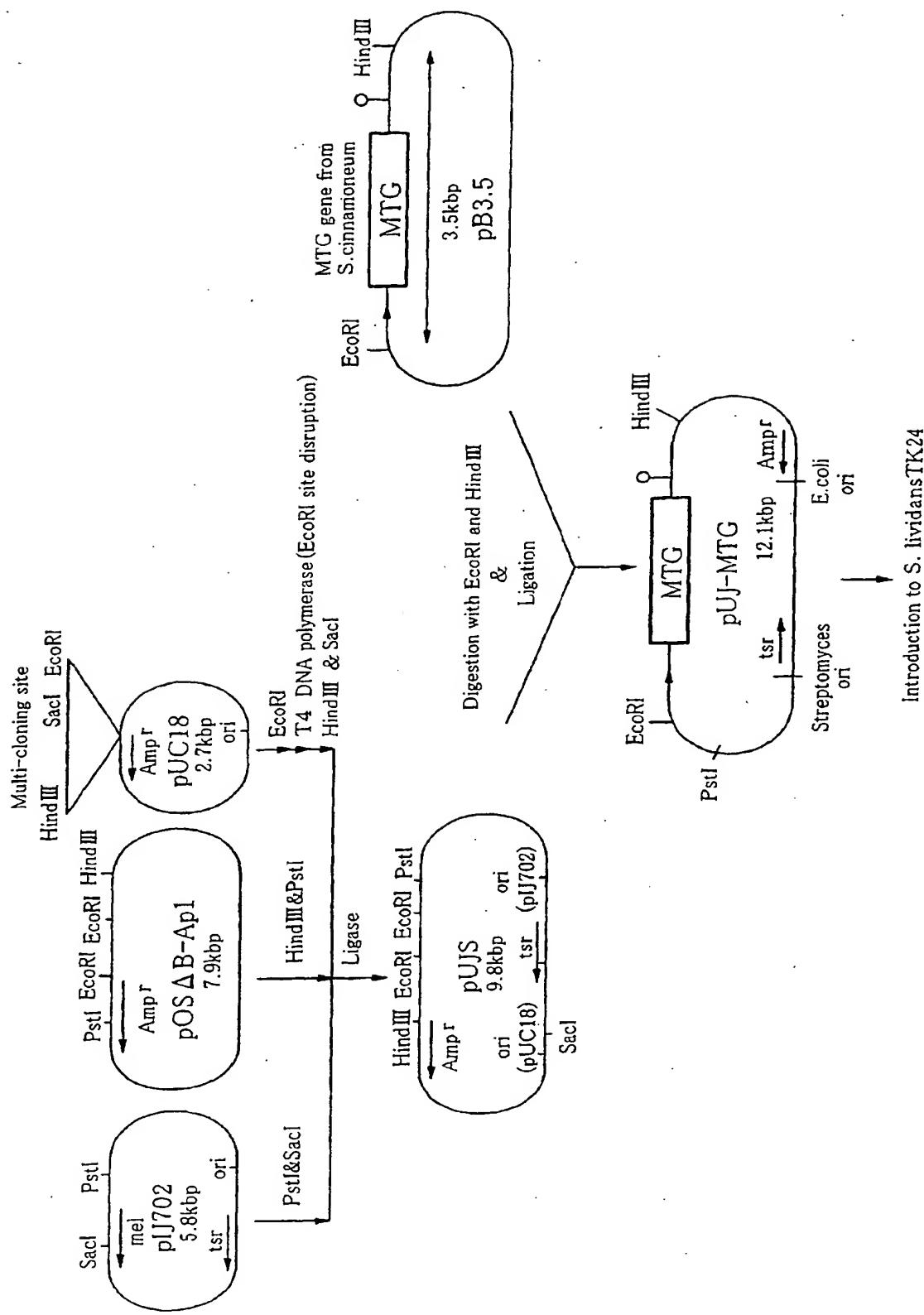
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FIG. 1



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/07135

A. CLASSIFICATION OF SUBJECT MATTER  
 Int.C1' C12N1/21, 9/10, 15/54 // (C12N1/21, C12R1:465)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 Int.C1' C12N1/21, 9/10, 15/54

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
 BIOSIS (DIALOG), WPI (DIALOG), SwissProt/PIR/GeneSeq

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, 481504, A1 (AMANO PHARMACEUTICAL CO., LTD.), 22 April, 1992 (22.04.92) & JP, 5-199883, A & US, 5420025, A & DE, 69116495, E	1-7
A	WASHIZU, Kinya et al., "Molecular Cloning of the Gene for Microbial Transglutaminase from Streptoverticillium and Its Expression in Streptomyces lividans", Bioscience, Biotechnology, and Biochemistry, January 23, 1994, Volume 58, Number 1, pages 82-87	1-7
A	WO, 96/06931, A1 (NOVO NORDISK A/S), 07 March, 1996 (07.03.96), especially, see pages 76-77, SEQ ID NO:2 & AU, 9532538, A & EP, 777726, A1 & NZ, 291414, A & JP, 10-504721, A & US, 6100053, A	1-7
A	DURAN, R. et al., "Purification, characterisation, and genecloning of transglutaminase from Streptoverticillium cinnamoneum CBS 683.68",	1-7

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document but published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 January, 2001 (12.01.01)	Date of mailing of the international search report 23 January, 2001 (23.01.01)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Faxsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/07135

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Biochimie, April, 1998, Volume 80, Number 4, pages 313-319 PASTERNACK, Ralf et al., "Bacterial pro-transglutaminase from Streptomyces mobaraense Purification, characterisation and sequence of the zymogen", European Journal of Biochemistry, November 1, 1998, Volume 257, Number 3, pages 570-576	1-7
A	EP, 889133, A2 (AJINOMOTO CO., INC.), 07 January, 1999 (07.01.99) & JP, 11-75876, A & CA, 2237041, A & US, 6013498, A & BR, 9802403, A & CN, 1253177, A	1-7

Form PCT/ISA/210 (continuation of second sheet) (July 1992)